METHOD OF MANUFACTURE OF A BIOACTIVE ARTICLE FOR TREATMENT OF DISEASE

RELATED APPLICATIONS

[001] This application claims priority under 35 U.S.C. § 119(e) of U.S. Provisional Application 60/149,744, METHOD OF MANUFACTURE OF A BIOACTIVE ARTICLE FOR TREATMENT OF DISEASE filed August 19, 1999, the entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

[002] This invention relates to methods of manufacturing an article useful as a device to treat disease. The article comprises a biocompatible enclosure and viable cells contained therein.

BACKGROUND OF THE INVENTION

[003] United States patent application Ser. No. 08/840,804 filed April 16, 1997, now U.S. Patent 5,972,332, (incorporated herein by reference) describes a means for treating a wound-site or other tissue in need of treatment with cells cultured on solid support material enclosed in a biocompatible enclosure. Methods for effecting such treatment means, including using transformed cells expressing a transgene, are described in U.S. Ser. No. 09/323,188, filed May 27, 1999 and U.S. Ser. No. 09/338,413, Attorney Docket No. UMV-1654CP, filed February 11, 2000. Methods for making an article of manufacture useful in the delivery of such treatments are described and claimed herein.

SUMMARY OF THE INVENTION

[004] This invention provides a process for manufacturing a bioactive means for treating a wound-site or other tissue in need of treatment comprising the steps of providing a plurality of substantially solid cell-support means; a biocompatible enclosure means; a vehicle compatible with cell viability; and cells. In different embodiments of the invention, the steps vary in sequence.

[005] This invention provides means for determining, at various stages of manufacture, (i) the viability of the cells, (ii) the distribution of viable cells on each one of the plurality of solid-support means; (iii) the distribution of viable cells as between the solid-support means and either the enclosure means or the vehicle; and (iv) the density of the cells on the solid cell-support means.

[006] This invention provides means for selecting and preparing the solid-support means and the enclosure means. This invention provides means for seeding the solid-support means with cells, and means for promoting proliferation of seeded cells. This invention provides means for storing the cell-seeded solid supports and means for preparing the enclosed cell-seeded solid supports for use.

[007] In some embodiments, the present invention provides methods for preparing an article comprising viable cells on an insoluble support comprising: a) providing an enclosure that is permeable to solutes; b) introducing into the enclosure an insoluble support material and sealing the enclosure; and c) introducing viable cells into the enclosure whereby the cells attach to the insoluble support material. In other embodiments, the cells are injected into the enclosure. In certain embodiments, the enclosure comprises a mesh material and the injection is performed through the mesh.

[008] In particular embodiments, the enclosure comprises a septum and the injection is performed through the septum. In other embodiments, the method further comprises freezing the enclosure containing the viable cells. In additional embodiments, the method further comprises thawing the enclosure containing the viable cells and maintaining the thawed enclosure in a tissue culture medium. In some embodiments, the method further comprises testing the viability of the cells by staining the cells with a dye that fluoresces upon binding of calcium and with a dye that fluoresces upon intercalation into DNA, then measuring the ratio of fluorescence of the calcium staining dye to the fluorescence of the DNA intercalating dye.

[009] In certain embodiments, the present invention provides methods for preparing an article comprising viable cells on an insoluble support comprising: a) providing an enclosure that is permeable to solutes but is impermeable to cells; b) introducing into the enclosure an insoluble support material and then sealing the enclosure in a biocompatible manner; and c) introducing viable cells into the sealed enclosure by injection. In particular embodiments, the method further comprises testing the viability of the cells by sampling

the cells within the enclosure and staining the cells with a dye that fluoresces upon binding of calcium and with a dye that fluoresces upon intercalation into DNA, then measuring the ratio of fluorescence of the calcium staining dye to the fluorescence of the DNA intercalating dye.

[010] In some embodiments, the present invention provides methods for preparing an article comprising viable cells on an insoluble support comprising: a) providing an enclosure that is permeable to solutes and to cells; b) introducing into the enclosure an insoluble support material having attached thereto a plurality of viable cells; c) immersing the enclosure containing the support material into a culture of viable cells, whereby the cells migrate into the enclosure and attach to the support material. In other embodiments, the method further comprises removing the enclosure from the culture and freezing the article. In additional embodiments, the support material is treated with a hydroxide solution prior to introducing the support material into the enclosure.

DETAILED DESCRIPTION OF THE INVENTION

[011] Any solid-support means capable of sustaining a living cell (within the meaning of the viability test disclosed herein) may be selected for use by the skilled practitioner of this invention. In preferred embodiments, solid supports will be selected to provide adequate surface area to accommodate cell attachment and growth to a sufficiency for the purposes of the invention. In preferred embodiments, also, solid supports will be selected to be of such a shape and size as to be substantially incapable of permeating the selected enclosure means. It is not intended, however, that such impermeability be achieved solely by the shape or size of the solid supports. Other properties, such as the degree of hydrophilicity and hydrophobicity, the electrical charge properties, and the lipophilicity or polarity of either the solid supports or the enclosure means may also be employed to achieve the preferred impermeability.

[012] To promote cell-seeding and/or cell viability, solid-support means treated or coated with biocompatible substances to render them more or less hydrophilic or hydrophobic, cationic or anionic, polar or non-polar are also contemplated to be within the scope of this invention.

[013] Any biocompatible enclosure means capable of substantially retaining the selected cell-support means may be selected by the skilled practitioner of this invention. P530

Natural (AET, Inc.) is a specific example. In preferred embodiments an enclosure means that is generally permeable to solutes will be selected. In certain embodiments an enclosure means that is permeable to cells in at least one direction will be preferred. In certain embodiments enclosure means that resist attachment of cells to one or both of their surfaces are preferred. In certain embodiments, the enclosure means must be sealable in such a way that a biocompatible seal results. In certain embodiments, the skilled practitioner will select an enclosure means and a sealing means such that the enclosure means and the seal remain useful for the purposes of the invention after being subjected to temperatures adequately high to sterilize the enclosure means or adequately low to preserve cell viability by means of freezing. The properties enumerated in the foregoing are exemplary and, in general, do not need to be combined in any particular aggregation to effect a useful embodiment of the invention. Thus, cells may be introduced into a pre-sealed enclosure by means of injection or by effecting immigration of cells through a cell-permeable enclosure means. Alternatively, cell-support means and cells may be introduced into a sealably open enclosure. The cell support means, moreover, need not be seeded with cells when the cell support means is introduced into the enclosure. Indeed, embodiments are contemplated wherein at least a portion of the cell-support means and at least a portion of the cells in each manufactured article are introduced into, and reside in, the enclosure in separate compartments. Compartmentalization may be achieved by a physical barrier such as a membrane, or by segregating the support means and the cells in separate vehicles, which vehicles are rendered immiscible by virtue of having been frozen separately, or suspended in immiscible gels.

[014] The skilled practitioner may select from a number of cell culture media known to support cell growth. The selection, for which the skilled practitioner will have ample guidance from the prior art, will depend upon the cell-type and specific conditions chosen to prepare the cell culture.

[015] The selection of cells depends upon the bioactive treatment intended. For example, keratinocytes derived from breast tissue, foreskin, abdominal full or split thickness skin, or the skin of cadavers are useful in the treatment of wounds. Other acceptable primary cell types include, without limitation, fibroblasts, melanocytes, endothelial cells, blood-borne cells, and osteocytes.

[016] Any cell transformed by the insertion of DNA by any means that results in a cell capable of secreting an expression product of the inserted DNA, or a product whose expression by the cell is promoted or enhanced by the insertion of the DNA is also suitable. The particular DNA to be selected will depend upon the treatment to be applied. An ample literature exists to provide guidance to the skilled practitioner in this regard.

EXAMPLES

[017] The following examples are by way of illustration and not limitation and are intended primarily to provide guidance to the skilled artisan in making and using the article.

[018] Preparation of Cells: A method of isolating keratinocytes, seeding solid support means with keratinocytes, and introducing cell-seeded support means into an enclosure is described in U.S. Patent 5,972,332. Similar methodology is described for a transformed cell line expressing a transgene in U.S. Patent Application Ser. no. 09/323,188 filed May 27, 1999 and in U.S. Ser. No. 09/338,413, filed February 11, 2000. Each referenced patent and application is incorporated herein in its entirety by reference. Virtually any other cell type that is mitotically competent in a cell culture medium could be selected by the skilled practitioner depending upon the specific purpose for which the subject article is to be used. Primary isolates may be seeded onto solid support means immediately, or passaged one or more times in cell culture before seeding. In the case of keratinocytes, the cell culture medium contains microgram per ml amounts of insulin, calcium (at concentrations lower than normal serum concentrations), microgram per ml amounts of a glucocorticoid, nanogram/ml amounts of epidermal growth factor, and nanogram/ml amounts of a saline extract of bovine pituitary.

[019] Preparation of Solid Support Means: A solid support means with the following features is selected:

(i) Preferably, the solid support means is substantially incapable of escaping the enclosure. In this example, macroporous beads made of polyethylene and silica, sized to be large enough to stay inside an enclosure made of Delnet™ (P530

Natural, AET, Inc.) but otherwise small enough to maximize the surface area that a plurality of the beads provides for cell attachment and cell growth, were selected.

(ii) The solid support means preferably has a higher avidity for cells than for the selected enclosure material or the culture medium. In this case, the selected beads were treated with a solution of sodium hydroxide to effect this result, presumably by rendering the beads more negatively charged than the enclosure material or the culture medium.

(iii) A solid support means that is non-toxic to the cells (within the limits of the viability test described herein) must be selected.

[020] In the instant example, the selected solid support means, macroporous microcarrier beads made of polyethylene and silica, were autoclaved at 121°C for 10 minutes in double distilled or microfiltered water. Following the autoclaving procedure, the beads were allowed to settle by gravity and were cooled. The supernatant water was then aspirated away from the beads. The beads were re-suspended in double distilled or microfiltered water and agitated by hand-swirling for a few seconds. The beads were again allowed to settle, the water aspirated and a final washing performed as above.

[021] The beads were next treated with a solution of 0.1N NaOH, a preferred concentration, but not critical. Treatment continued at room temperature overnight. Thereafter the washing procedure set out above was repeated five times. The pH of the final suspension was measured with a conventional device at less than about 8. The beads were then transferred to sterile culture medium, at which point they are ready for seeding.

[022] Preparation of the Enclosure: Variations in the procedure result in several embodiments of the final article.

(i) A sheet of Delnet™, approximately 3x6 inches was folded once into a square shape and heat-sealed on two sides using a heat-sealing unit (Impulse™ manufactured by American International Electric Co.) to create a bag-like structure. Next, beads prepared as described above were introduced into the bag through the unsealed

side. Approximately 1 ml of gravity-packed beads was introduced into the bag. The open side of the bag was then heat-sealed as above. The article was then immediately placed in culture medium, heat-sterilized at 121°C for 30 minutes and stored in the culture medium for use in subsequent steps.

(ii) The bag prepared as described above, after equilibrating in culture medium at 37°C for 4 days or more was inoculated with a primary culture of more than about one million keratinocytes per ml of gravity-packed beads and less than about two million keratinocytes per ml of gravity-packed beads. The keratinocytes were suspended in culture medium in a 3cc syringe and injected through the Delnet material using an 18 ga. needle. The injected bag was then incubated, using conventional tissue culture apparatus, in approximately 1 ml of culture medium for 4 days at 37°C under 95%O_2, 5%CO_2. The article was then ready for use. Alternatively, the bag and its contents may be frozen and used at a later time, after thawing.

(iii) A sheet of Delnet™ is fashioned into an open bag-like structure and sterilized. Cells pre-seeded onto sterile, equilibrated solid-support means are introduced into the bag and the bag is then sealed by means that are non-toxic to the cells. The sealed bag is then incubated as described above or for a time sufficient to permit growth of cells to a pre-determined number and then put to use or frozen for use at a later time.

(iv) A sheet of Delnet™ is fashioned into an open bag-like structure and sterilized. Cells in suspension and, separately, sterile, equilibrated solid-support means are introduced into the bag and the bag is then sealed by means that are non-toxic to cells. The sealed bag is then incubated as described above and put to use or frozen for use at a later time.

(v) Cells in suspension and, separately, sterilized solid-support means are introduced into a sterilized, entirely sealed enclosure. The enclosure is then incubated as described above and put to use or frozen for use at a later time. The frozen article

is made useable by thawing and, using conventional tissue culture apparatus, incubating the article in culture medium for a sufficient time to permit seeding and growth of cells to a pre-determined number.

(vi) Cells in suspension are introduced into either an open bag or an entirely sealed bag and frozen. Separately, an equilibrated, sterile solid-support means is introduced into the same bag, sealed by an appropriate means if necessary, and frozen. At a later time, the bag is thawed and, using conventional tissue culture apparatus, incubated in culture medium for a sufficient time to permit seeding and growth of cells to a pre-determined number. The article is then ready for use.

(vii) An enclosure prepared as in (i) above is co-incubated with cells. However, the cells are not injected into the enclosure, but migrate into the enclosure. When the extent of cell immigration is sufficient to effect seeding of the solid support means, incubation of the enclosure and its contents continues, with or without replacement of the medium with cell-free medium, to permit growth of cells on the solid-support means to a pre-determined number. The Article is then put to use or frozen for use at a later time. The frozen article is made useable by thawing and, using conventional tissue culture apparatus, incubating the article in culture medium for a sufficient time to permit seeding and growth of cells to a pre-determined number.

[023] Growing the Cells: Whether or not grown within or without the enclosure, cells were added to beads at a density of less than about two million cells per 1 ml of gravity-packed beads. Cells were grown in an incubator with slow agitation in a roller bottle. Cells attached to the beads within about 6 hours. The incubation medium was changed every other day. The temperature ranged between 36 and 38 °C. and the medium was gassed with 5%CO_2-95%O_2.

[024] Procedure for Assessing Viability of Cells in Enclosure:

Testing the viability of Cells:

Reagents:

1 μM calcein AM* + 2 μM EthD-1* in D-PBS

1 μM calcein AM in D-PBS

2 μM EthD-1 in D-PBS

Dulbecco's Buffered Phosphate Saline (D-PBS)

* Molecular Probes, Live/Dead® Viability/Cytotoxicity Kit (L-3224)

[025] Solution Preparation for experimental and control cell samples:

Use within 24 hours of making

Solution 1. Combination Solution (Live/Dead cells)

Add 20 μl EthD-1 to 10 ml D-PBS

Vortex to mix

Add 5 μl calcein to EthD-1 solution

Vortex to mix

Solution 2. Calcein solution (live cells)

Add 5 μl calcein to 10 ml D-PBS

Vortex to mix

Solution 3. EthD-1 solution (dead cells)

Add 20 μl EthD-1 to 10 ml PBS

Vortex to mix

[026] Experimental Sample Preparation:

Open a bag of cells/beads and place into a 35 mm Petri dish.

Aspirate the media.

Rinse cells/beads immediately with 3 ml of D-PBS.

Place aliquots of cells/beads into 4 Petri dishes for different treatments.

Aspirate the D-PBS.

Add enough of the combination solution (solution 1) to cover the cells/beads.

Incubate Petri dish at 37 degrees Celsius for 20 minutes.

View Petri dish under fluorescence microscope to confirm the cells have stained appropriately. (live cells: ex/em ~485/~530; dead cells: ex/em ~530/~645)

Randomly pick cells/beads from Petri dish and place into a 96 well plate.

[027] Control Samples Preparation:

[028] Plain Bead Controls:

Take plain prepared beads and place approximately 500 µl into 2 Petri dishes.

Aspirate soaking solution.

Rinse plain prepared beads with 3 ml of D-PBS.

Aspirate the D-PBS.

Add enough of Solution 2 to one Petri dish and Solution 3 to the remaining Petri dish to cover the beads.

Incubate Petri dishes at 37 degrees Celsius for 20 minutes.

Randomly pick beads and place into a 96 well plate.

Also take plain prepared beads (no solution treatments), rinsed in D-PBS and place into a 96 well plate.

[029] Cells/bead Controls:

Take one of the cell/bead aliquot dishes and aspirate the D-PBS.

Add enough of the Calcein solution (solution 2) to cover the cells/beads.

Take another of the cell/bead aliquot dish and aspirate the D-PBS.

Add enough of the EthD-1 solution (solution 3) to cover the cells/beads.

Take a third cell/bead aliquot dish and aspirate the D-PBS.

Add enough D-PBS to cover the cells/beads.

Incubate all Petri dishes at 37 degrees Celsius for 20 minutes.

Randomly pick beads and place into a 96 well plate.

[030] 96 well plate set up:

Pipet 100 µl D-PBS into all wells, 2-3 beads per well

Add plain bead controls, cells/beads controls and experimental samples to multiple wells for analysis.

[031] Microplate Reader Set Up:

Follow the instrument's procedure manual for setting up the reader for the appropriate filters and running the assay.

Calcein is excited using a fluorescein filter, 485 ± 10 nm and an emission filter of 530 ± 12.5nm.

Ethidium homodimer-1 (EthD-1) is excited with a rhodomine filter, 530 ± 12.5 nm and an emission filter of 645 ± 20 nm.

[032] Fluorescence Measurements:

Run the assay first utilizing the Calcein set of filters then repeat the assay with the Ethidium filter set. Background fluorescence (plain bead control) readings are subtracted from experimental sample wells. Viability = ratio of live cells values (Calcein assay) to dead cells values (Ethidium assay). Note: The minimum detectable number of cells per well is usually between 200 and 500. The maximum usable number of cells per well is on the order of 10^6. Reference Live/Dead® Viability Kit, Molecular Probes Inc., Information sheet.

What is claimed is:

1. A method for preparing an article comprising viable cells on an insoluble support comprising:

a) providing an enclosure that is permeable to solutes;

b) introducing into the enclosure an insoluble support material and sealing the enclosure; and

c) introducing viable cells into the enclosure whereby the cells attach to the insoluble support material.

2. The method of claim 1 wherein the cells are injected into the enclosure.

3. The method of claim 2, wherein the enclosure comprises a mesh material and the injection is performed through the mesh.

4. The method of claim 2, wherein the enclosure comprises a septum and the injection is performed through the septum.

5. The method of claim 1, further comprising freezing the enclosure containing the viable cells.

6. The method of claim 5, further comprising thawing the enclosure containing the viable cells and maintaining the thawed enclosure in a tissue culture medium.

7. The method of claim 1, further comprising testing the viability of the cells by staining the cells with a dye that fluoresces upon binding of calcium and with a dye that fluoresces upon intercalation into DNA, then measuring the ratio of fluorescence of the calcium staining dye to the fluorescence of the DNA intercalating dye.

8 A method for preparing an article comprising viable cells on an insoluble support comprising:

a) providing an enclosure that is permeable to solutes but is impermeable to cells;

b) introducing into the enclosure an insoluble support material and then sealing the enclosure in a biocompatible manner;

c) introducing viable cells into the sealed enclosure by injection.

9. The method of claim 8, further comprising testing the viability of the cells by sampling the cells within the enclosure and staining the cells with a dye that fluoresces upon binding of calcium and with a dye that fluoresces upon intercalation into DNA, then measuring the ratio of fluorescence of the calcium staining dye to the fluorescence of the DNA intercalating dye.

10. A method for preparing an article comprising viable cells on an insoluble support comprising:

a) providing an enclosure that is permeable to solutes and to cells;

b) introducing into the enclosure an insoluble support material having attached thereto a plurality of viable cells;

c) immersing the enclosure containing the support material into a culture of viable cells, whereby the cells migrate into the enclosure and attach to the support material.

11. The method of claim 10, further comprising removing the enclosure from the culture and freezing the article.

12. The method of claim 10, wherein the support material is treated with a hydroxide solution prior to introducing the support material into the enclosure.

ABSTRACT

[033] A method of manufacturing an article comprising seeding and growing living cells on a solid support in an enclosure by means of injecting cells into the enclosure or allowing the immigration of cells into the enclosure is described. A procedure for testing the viability cells at stages of the manufacturing process is described.